Extremophiles as a source for novel enzymes

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Microbial life does not seem to be limited to specific environments. During the past few decades it has become clear that microbial communities can be found in the most diverse conditions, including extremes of temperature, pressure, salinity and pH. These microorganisms, called extremophiles, produce biocatalysts that are functional under extreme conditions. Consequently, the unique properties of these biocatalysts have resulted in several novel applications of enzymes in industrial processes. At present, only a minor fraction of the microorganisms on Earth have been exploited. Novel developments in the cultivation and production of extremophiles, but also developments related to the cloning and expression of their genes in heterologous hosts, will increase the number of enzyme-driven transformations in chemical, food, pharmaceutical and other industrial applications.

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Current Opinion in Microbiology 2003, **6**:213–218

This review comes from a themed issue on
Ecology and industrial microbiology
Edited by Bernard Witholt and Eugene Rosenberg

1369-5274/03/$ – see front matter

DOI 10.1016/S1369-5274(03)00060-2

Introduction

Driven by increasing industrial demands for biocatalysts that can cope with industrial process conditions, considerable efforts have been devoted to the search for such enzymes. Compared with organic synthesis, biocatalysts often have far better chemical precision, which can lead to more efficient production of single stereoisomers, fewer side reactions and a lower environmental burden [1]. Despite the fact that to date more than 3000 different enzymes have been identified and many of these have found their way into biotechnological and industrial applications, the present enzyme toolbox is still not sufficient to meet all demands. A major cause for this is the fact that many available enzymes do not withstand industrial reaction conditions. As a result, the characterization of microorganisms that are able to thrive in extreme environments has received a great deal of attention: such extremophiles are a valuable source of novel enzymes [2,3].

Extreme conditions can refer to physical extremes (e.g. temperature, pressure or radiation), but also to geochemical extremes such as salinity and pH (Table 1). Most of the extremophiles that have been identified to date belong to the domain of the Archaea. However, many extremophiles from the eubacterial and eukaryotic kingdoms have also been recently identified and characterized.

The notion that extremophiles are capable of surviving under non-standard conditions in non-conventional environments has led to the assumption that the properties of their enzymes have been optimized for these conditions. Indeed, data for a considerable fraction of the enzymes that have been isolated and functionally characterized from extremophiles support this assumption.

In this review, I present and discuss recent examples of the discovery, isolation and application of enzymes from extremophiles.

Extremophiles

As illustrated in Table 1, the classification of 'extreme environments' refers to a wide variety of different conditions to which microorganisms have adapted. The biocatalysts obtained from these microorganisms could be applicable in similarly diverse conditions (Table 1). For the degradation of polymers such as chitin, cellulose or starch, enzymes that are active at and resistant to high temperatures are often preferred. Under these conditions the solubility and, consequently, the accessibility of the substrate is improved. Alternatively, if one needs to perform a stereospecific modification of a compound for the synthesis of a pharmaceutically relevant product in organic solvents, very different prerequisites apply to the biocatalysts. As salt is known to reduce water activity, enzymes from halophilic microorganisms could be the most suitable choice for application in nonaqueous media [4,5•]. This diversity of environments to which different extremophiles have adapted offers many exciting opportunities for a variety of applications.

Thermophiles

Thermophilic extremophiles have attracted most attention. In particular extremophilic proteases, lipases and polymer-degrading enzymes, such as cellulases, chitinases and amylases have found their way into industrial applications (Table 1). The reasons to exploit enzymes that are stable and active at elevated temperatures are obvious. At elevated temperatures the solubility of many reaction components, in particular polymeric substrates, is significantly improved. Moreover, the risk of contamination, leading to undesired complications, is reduced at higher temperatures.

Table 1

Classification of extremophiles and examples of applications of some of their enzymes.

Type	Growth characteristics	Enzymes	Applications
Thermophiles	Temp >80°C (hyperthermophile) and 60–80°C (thermophile)	Proteases	Detergents, hydrolysis in food and feed, brewing, baking
		Glycosyl hydrolases (e.g. amylases, pullulanase, glucoamylases, glucosidases, cellulases, xylanases)	Starch, cellulose, chitin, pectin processing, textiles
		Chitinases	Chitin modification for food and health products
		Xylanases	Paper bleaching
		Lipases, esterases	Detergents, stereo-specific reactions (e.g. *trans*-esterification, organic biosynthesis)
		DNA polymerases	Molecular biology (e.g. PCR)
		Dehydrogenases	Oxidation reactions
Psychrophiles	Temp <15°C	Proteases	Detergents, food applications (e.g. dairy products)
		Amylases	Detergents and bakery
		Cellulases	Detergents, feed and textiles
		Dehydrogenases	Biosensors
		Lipases	Detergents, food and cosmetics
Halophiles	High salt, (e.g. 2–5 M NaCl)	Proteases	Peptide synthesis
		Dehydrogenases	Biocatalysis in organic media
Alkaliphiles	pH >9	Proteases, cellulases	Detergents, food and feed
Acidophiles	pH <2–3	Amylases, glucoamylases	Starch processing
		Proteases, cellulases	Feed component
		Oxidases	Desulfurization of coal
Piezophiles	Pressure-loving; up to 130 MPa	To be defined	Food processing and antibiotic production

The structural features of thermophilic extremozymes has attracted much attention. Several three-dimensional structures have been solved and compared with those of mesophilic counterparts, with the ultimate goal of elucidating the mechanisms underlying thermostability [6,7•,8•]. In a comprehensive study, 10 thermophilic and hyperthermophilic serine hydroxymethyltransferases were compared with 53 mesophilic homologs [9•]. Structural alignment and homology modeling was applied to identify the different mechanisms involved in thermal stability. For this enzyme class, it was concluded that stability was achieved by a combination of increased surface charge, increased protein core hydrophobicity and replacement of exposed 'thermolabile' amino acids.

Psychrophiles

More recently, enzymes from psychrophiles have become interesting for industrial application, partly because of ongoing efforts to decrease energy consumption. For example, there is an increasing desire to apply psychrophilic enzymes in detergents. With such enzymes it becomes feasible to develop laundry applications that can be performed at lower temperatures. For such processes, psychrophilic proteases, amylases or lipases have great commercial potential. The pulp and paper industry is also interested in polymer-degrading enzymes that are active at lower temperatures. Several food processing applications would also benefit from the availability of low temperature enzymes.

A characteristic feature of many enzymes from psychrophiles is the correlation of high catalytic activity and low thermal stability at moderate temperatures, which can be partly explained by the increased flexibility of the molecule, compared with mesophilic and thermophilic enzymes. This adaptation of the enzymes to low temperatures has been the subject of several studies [10–15]. It has been assumed that increased flexibility is correlated with decreased stability and a delicate balance between stability and activity is indeed often observed. Nevertheless, there is an increasing number of examples from nature as well as from protein engineering studies showing that the structural features involved in stability or activity can be very different and act independently [16–19]. A good overview of present knowledge on the properties of enzymes from psychrophiles and their applications can be found in two recent reviews [20••,21].

Halophiles

Halophiles can survive in hypersaline habitats by their ability to maintain osmotic balance. They accumulate salts such as sodium or potassium chloride (NaCl or KCl), up to concentrations that are isotonic with the environment. As a result, proteins from halophiles have to cope with very high salt concentrations (e.g. KCl concentrations of ~4 M and NaCl concentrations of >5 M) [22,23]. The enzymes have adapted to this environmental pressure by acquiring a relatively large number of negatively charged amino acid residues on their surfaces to prevent precipitation. Consequently, in surroundings with lower salt concentrations the solubility of halophilic enzymes is often very poor, which could limit their applicability [24]. However, this property has been taken advantage of by

applying halophilic enzymes in aqueous/organic and non-aqueous media [25]. For example, an extracellular protease from *Halobacterium halobium* has been exploited for efficient peptide synthesis in water/N′-N′-dimethylformamide [26].

Recently, a *p*-nitrophenylphosphate phosphatase (*p*-NPPase) from *Halobacterium salinarum* was used in an organic medium at very low salt concentrations after entrapping the enzyme in reversed micelles [5•]. Under these conditions *p*-NPPase was active and stable. Similar observations were made with a halophilic malate dehydrogenase [27]. Exploitation of reversed micelles in combination with halophilic enzymes is likely to result in the development of novel applications for these enzymes [28].

Alkaliphiles/acidophiles

Enzymes from microorganisms that can survive under extreme pH could be particularly useful for applications under highly acidic or highly alkaline reaction conditions, for example in the production of detergents. However, one of the striking properties of acidophilic and alkaliphilic microorganisms is their ability to maintain a neutral pH internally, and so the intracellular enzymes from these microorganisms do not need to be adapted to extreme growth conditions. However, this does not account for extracellular proteins, which have to function in low or high pH environments in the case of acidophiles and alkaliphiles, respectively.

Proteases, amylases, lipases and other enzymes that are resistant to and active at high pH and high chelator concentrations of modern detergents are desirable. This has prompted the screening of alkaliphilic bacteria and Archaea for their ability to produce such enzymes. By these means, several useful enzymes have already been identified and obtained. Combinations of homology-based PCR and activity screening have been applied to screen for and detect alkaline proteases in a collection of thermoacidophilic archaeal and bacterial strains isolated from hot environments [29]. In an alternative approach, alkaliphilic bacilli that could grow at >pH 9 were used as a source for oxidation-resistant alkaline proteases [30].

Polymer-hydrolysis related processes have also initiated the search for biocatalysts from acidophiles. Several enzymes used for starch-hydrolysis (e.g. amylases, pullulanases, glucoamylases and glucosidases that are active at low pH) have been isolated [31,32].

Piezophiles

It is thought that pressure does not exert a major selective force on protein function [33]. This assumption is based on the consideration that pressures exceeding 400 MPa are needed to induce the denaturation of single-chain proteins. In view of that, it should be noted that even microorganisms that live in the deep sea are not exposed to pressures that exceed 120 MPa [33]. Under those conditions, their enzymes do not need specific pressure-related adaptations. However, there are some examples of the specific stabilization of proteins by increased pressure [34,35]. Pressure-resistant proteins could be of use, in particular for food production, where high pressure is applied for processing and the sterilization of food materials [36]. The biotechnological opportunities for piezophiles have recently been reviewed in detail [37].

Other extremophiles

At present, it is clear that no matter how extreme the conditions are at defined locations on Earth there is a fair chance that microbes will be able to survive. Additional examples further to those described above are microorganisms that grow in the presence of high metal concentrations (metallophiles), at high radiation levels (radiophiles), or under oxygen deprivation (microaerophiles). The biotechnological application of enzymes from such extremophiles is not always obvious. Nevertheless, in view of the great potential of biocatalysis it is very likely that new concepts will be developed that will result in the application of enzymes from these and other extremophiles in industrial processes.

Extreme genomes

The growing number of genomes available from extremophiles will greatly aid the discovery and identification of useful novel enzymes. Currently, at least 10 genomes from Archaea have been entirely sequenced and another 12 are close to completion [38]. In addition, a significant number of genomes from extremophilic bacteria have and are currently being elucidated. Illustrative of the large potential of extremophiles as a source for novel enzymes is the observation that was made after determining the 1.56 Mb genome of *Thermoplasma acidophilum* [39]. In this genome, approximately 1500 open reading frames (ORFs) were identified. For 240 of these ORFs no recognizable homologs could be found in DNA databases available in the public domain. It is tempting to assume that a considerable fraction of the proteins encoded by these 'unknown' ORFs is responsible for the unique ability of this microorganism to thrive under extreme conditions. In addition, among this group of 'unknown' genes there could be a considerable amount of genes that encode enzymes that are of industrial interest. This growing wealth of data will therefore be useful for the identification of enzymes that have not been detected by functional screening procedures. This is illustrated by the observation that within the genomes of archaeal *Methanosarcina* spp multiple homologs for enzymes involved in methanogenesis were present [40]. Expression of the individual genes was shown to be strongly dependent on the composition of the growth medium. Thus, functional screening could have prevented the identification of all potentially useful gene products.

Production of enzymes from extremophiles

Functional activity screening and mining of DNA data can be very helpful for the identification of useful enzymes. However, the identification of potentially useful enzymes is only a first step. To take full advantage of this knowledge, the identified enzymes have to be made available for in-depth assessment of the biocatalytic properties and application tests. There are two strategies for the production of enzymes from extremophiles. First, production of the biocatalysts can be optimized by increasing the biomass production of the extremophile. Alternatively, the gene encoding the biocatalyst can be cloned and expressed in a suitable host.

Extremophile biomass production

Specialized equipment is often a prerequisite to obtain sufficient amounts of a specific biocatalyst directly from the extremophile. In addition, tailor-made fermentation processes will have to be developed and many issues will have to be addressed in the cultivation of extremophiles [41••]. Biomass production can be improved either by optimization of the medium composition or by varying the specific fermentation procedures (e.g. fed-batch, cell recycling or continuous cultivation). In addition, special bioreactors such as gas-lift fermenters can and often have to be used. For example, specific corrosion-resistant bioreactors had to be developed in the cultivation of extreme halophiles for the production of polyglutamic acid (PGA) [42]. Other specific requirements that often have to be met are related to the high pressures that are characteristic of many environments from which the extremophiles are isolated such as the deep sea [43].

Gene cloning and overproduction

At present, the fermentation or production technology for extremophiles is not considered to be standard procedure. Therefore, other ways of making useful biocatalysts available from these sources have to be developed. The cloning and expression of encoding genes in mesophilic hosts can be an attractive alternative method. A major portion of the extremozymes that have found their way into industrial applications are produced using standard *Escherichia coli* expression systems (e.g. Table 2 in [22], [44]).

Genetic tools for *E. coli* expression are very well developed and many plasmid vectors and inducible gene expression systems are available. In particular, the production of thermophilic enzymes is facilitated by the efficient denaturation of most *E. coli* proteins following a short heat treatment [45–47]. However, the well-known phenomenon of inclusion-body formation by overexpressed protein in *E. coli*, could force one to search for alternative hosts. Fortunately, there is an increasing number of alternative hosts available, including bacterial systems such as *Bacillus*, *Pseudomonas*, *Lactobacillus*, *Lactococcus* and eukaryotic systems such as *Pichia*, *Kluyveromyces*, *Candida* and *Hansenula*. The objectives of the expression should be kept in mind in selecting a specific heterologous host system. For example, if the enzyme needs glycosylation for biological activity the selection of a bacterial host should be avoided, whereas for the production of an extracellular enzyme a bacterial host such as *Bacillus* is likely to be the obvious choice. A drawback of most *Bacillus*-based systems is the relatively high level of endogenous protease production by the host, which often leads to degradation of the heterologous gene product of interest. Recently, an extracellular protease-deficient *B. brevis* system was developed that results in better production levels of heterologous proteins [48].

Several of the expression systems mentioned above are applicable for the construction of genome libraries. In general, *E. coli* has been the host of choice for these applications [44,46,49]. Nevertheless, in view of the risk of inclusion-body formation and the fact that *E. coli* has poor protein export capabilities, alternatives should also be considered.

The availability of good cloning and expression systems can also be used for improving and tailoring biocatalytic properties of extremozymes by protein engineering and random mutagenesis. In particular, directed enzyme evolution is heavily dependent on the efficiency of such systems. Often, large libraries (10^4–10^7 variants) have to be screened to obtain variants with the desired function ([50,51] and references therein).

Conclusions and perspectives

Extremozymes have a great economic potential in many industrial processes, including agricultural, chemical and pharmaceutical applications. Many consumer products will increasingly benefit from the addition or exploitation of extremozymes. The toolbox to select and make such enzymes available is steadily expanding. It has been suggested that less than 10% of the organism in a defined environment will be cultivatable, and so further improvement of gene expression technologies (e.g. by the development of novel and improved heterologous host systems), will accelerate the exploration of microbial diversity. It is now possible to construct gene expression libraries from the most diverse sources. If such libraries are screened with fast and accurate detection technologies many new extremozymes will be discovered in the years to come. These extremozymes will be used in novel biocatalytic processes that are faster, more accurate, specific and environmentally friendly. Concurrent developments of protein engineering and directed evolution technologies will result in further tailoring and improving biocatalytic traits, which will increase the application of enzymes from extremophiles in industry.

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